IN THE SPECIFICATION:

Please delete the paragraph at page 1, lines 4-7.

Please insert the following new paragraph at page 1, immediately below the title:

-- **CROSS REFERENCE TO RELATED APPLICATIONS**

This application is a divisional (and claims the benefit of priority under 35 USC 120) of U.S. application serial no.09/978,758, filed October 16, 2001, which claims priority to and is a continuation-in-part of PCT/JP01/01082, filed February 15, 2001, which claims priority from Japanese Application 2000-43506, filed February 16, 2000, and Japanese Application No. 2000-374593, filed December 8, 2000. The disclosures of the prior applications are considered part of (and are incorporated herein by reference in their entirety) the disclosure of this application.--

-continued

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What is claimed is:

1. A substantially pure protein having activity of (R)-2-octanol dehydrogenase and encoded by a polynucleotide of (a) to (d) below:

(a) a polynucleotide comprising the nucleotide sequence of SEQ ID NO:1,

(b) a polynucleotide encoding a protein comprising the amino acid sequence of SEQ ID NO:2,

(c) a polynucleotide hybridizing under stringent conditions with a polynucleotide comprising the nucleotide sequence of SEQ ID NO:1, and

(d) a polynucleotide encoding an amino acid sequence having not less than 70% homology to the amino acid sequence of SEQ ID NO:2.

2. A substantially pure polypeptide comprising the amino acid sequence of SEQ ID NO:2.

3. A substantially pure polypeptide comprising an amino acid sequence at least 70% identical to SEQ ID NO:2, wherein said polypeptide has (R)-2-octanol dehydrogenase activity.

4. The polypeptide of claim 3, wherein said amino acid sequence is at least 80% identical to SEQ ID NO:2.

5. The polypeptide of claim 3, wherein said amino acid sequence is at least 90% identical to SEQ ID NO:2.

6. The polypeptide of claim 3, wherein said amino acid sequence is at least 95% identical to SEQ ID NO:2.

7. A substantially pure polypeptide comprising the amino acid sequence of SEQ ID NO:2 with up to 50 conservative amino acid substitutions, wherein said polypeptide has (R)-2-octanol dehydrogenase activity.

8. The polypeptide of claim 7, wherein the number of conservative amino acid substitutions is up to 30.

9. The polypeptide of claim 7, wherein the number of conservative amino acid substitutions is up to 10.

10. The polypeptide of claim 7, wherein the number of conservative amino acid substitutions is up to 3.

11. An isolated polypeptide comprising the amino acid sequences of SEQ ID NO: 3, SEQ ID NO: 4, and SEQ ID NO: 5, wherein said polypeptide has (R)-2-octanol dehydrogenase activity.

12. The polypeptide of claim 11, wherein the polypeptide is derived from a microorganism of the genus Pichia.

13. An isolated (R)-2-octanol dehydrogenase enzyme comprising the amino acid sequences of SEQ ID NO: 3, SEQ ID NO: 4, and SEQ ID NO: 5, wherein said enzyme has an oxidizing activity that 1) produces ketone by oxidizing alcohol using oxidized form of β-nicotinamide adenine dinucleotide as a coenzyme; and 2) of the two optical isomers of 2-octanol, preferentially oxidizes (R)-2-octanol.

14. The enzyme of claim 13, wherein said oxidizing activity has an optimal pH of about 8.0 to about 11.0.

15. The enzyme of claim 13, wherein said oxidizing activity shows higher activity on secondary alcohols than on primary alcohols.

16. The enzyme of claim 15, wherein said oxidizing activity shows significantly higher activity on (R)-2-octanol than on 2-propanol.

17. An isolated (R)-2-octanol dehydrogenase enzyme comprising the amino acid sequences of SEQ ID NO: 3, SEQ ID NO: 4, and SEQ ID NO: 5, wherein said enzyme has a reducing activity that produces 1) alcohol by reducing ketone using reduced form of β-nicotinamide adenine dinucleotide as a coenzyme, and 2) (S)-4-halo-β-hydroxybutyric acid esters by reducing 4-haloacetoacetic acid esters.

18. The enzyme of claim 17, wherein said reducing activity has an optimal pH of about 5.0 to about 6.5.

19. An isolated (R)-2-octanol dehydrogenase derived from *Pichia finlandica* having the following physicochemical properties (1) to (4):

(1) action

i) the enzyme produces ketone by oxidizing alcohol using an oxidized form of β-nicotinamide adenine dinucleotide as a coenzyme, and

ii) the enzyme produces an alcohol by reducing a ketone using a reduced form of β-nicotinamide adenine dinucleotide as a coenzyme;

(2) substrate specificity

i) of the two optical isomers of 2-octanol, the enzyme preferentially oxidizes (R)-2-octanol, and

ii) the enzyme produces (S)-4-halo-3-hydroxybutyric acid esters by reducing 4-haloacetoacetic acid esters;

(3) optimal pH

optimal pH for the oxidation reaction ranges from 8.0 to 11.0, and that for the reduction reaction ranges from 5.0 to 6.5;

(4) substrate specificity

i) the enzyme shows higher activity on secondary alcohols than on primary alcohols, and

ii) the enzyme shows significantly higher activity on (R)-2-octanol than on 2-propanol.

20. A substantially pure polypeptide consisting of the amino acid sequence of SEQ ID NO:2.

21. A substantially pure polypeptide consisting of the amino acid sequence of SEQ ID NO:2 and having up to 10 amino acid deletions, additions, insertion, or substitutions, wherein said polypeptide has (R)-2-octanol dehydrogenase activity.

22. The substantially pure polypeptide of claim 21, having up to 3 amino acid deletions, additions, insertion, or substitutions.

* * * * *

synthesizing (S)-4-halo-3-hydroxybutyric acid esters using these enzymes requires addition and regeneration of NADPH, which is expensive and chemically unstable, and is industrially disadvantageous.

- Some reductases derived from baker's yeast (D-enzyme-1, D-enzyme-2, J. Am. Chem. Soc., 107:2993-2994, 1985)
- Aldehyde reductase 2 derived from *Sporobolomyces salmonicolor* (Appl. Environ. Microbiol., 65:5207-5211, 1999)
- Keto pantothenic acid ester reductase derived from *Candida macedoniensis* (Arch. Biochem. Biophys., 294:469-474, 1992)
- 4-Chloroacetoacetic acid ethyl ester reductase derived from *Geotrichum candidum* (Enzyme Microb. Technol. 14, 731-738, 1992)
- Carbonyl reductase derived from *Candida magnoliae* (WO 98/35025)
- Carbonyl reductase derived from *Kluyveromyces lactis* (JP-A Hei 11-187869)
- β-Ketoacyl-acyl carrier protein reductase as one of fatty acid synthases type II (JP-A 2000-189170)

Although 3α-hydroxysteroid dehydrogenase (JP-A Hei 1-277494), glycerol dehydrogenase (Tetrahedron Lett. 29, 2453-2454, 1988), and alcohol dehydrogenase derived from *Pseudomonas sp.* PED (J. Org. Chem., 57:1526-1532, 1992) are known as reductases using reduced form of nicotinamide adenine dinucleotide (NADH) as a electron donor, these enzymes are industrially disadvantageous because the activity of reaction for synthesizing (S)-4-halo-3-hydroxybutyric acid esters is low.

As indicated above, known methods for producing (S)-4-halo-3-hydroxybutyric acid esters using microorganisms and enzymes were not satisfactory in some respects such as optical purities, yields, activities, etc. These problems have made known methods difficult for industrial use.

On the other hand, (R)-propoxybenzene derivatives (JP-A Hei 02-732) are useful compounds as intermediates in synthesizing medicines, especially, optically active substances of ofloxacin ((S)-(-)-9-fluoro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-2,3-dihydro-7H-pyrido[1,2,3-de][1,4]benzooxazine-6-carboxylic acid, JP-A Sho 62-252790), which is synthetic antibacterial drugs. How to get (to synthesize or separate) optically pure enantiomers of these compounds is industrially important problem.

Asymmetric acylation of racemates of propoxybenzene derivatives using lipase and esterase (JP-A Hei 03-183489) is known as a method for producing (R)-propoxybenzene derivatives. In this method, a process to separate remaining raw materials and acylated products after acylation of (R) form and a process to deacylate the acylated products are required. Therefore, this known method is inappropriate for industrial use because these processes are complicated.

The method for asymmetric reduction of acetonyloxybenzene derivatives using microorganisms has been also reported. However, this known method is inappropriate for industrial use because optical purities of (R)-propoxybenzene derivatives produced is as low as 84 to 98% (JP-A Hei 03-183489) or 8.8 to 88.4% (JP-A Hei 05-68577) and because the concentration of substrate is also as low as 0.1 to 0.5%. As the method in which high optical purities can be obtained by asymmetric reduction, the method using carbonyl reductase produced by *Candida magnoliae* (JP-A 2000-175693) was reported to synthesize (R)-propoxybenzene derivatives whose optical purities are 99% or more. However, this carbonyl reductase uses NADPH as a coenzyme. Therefore, synthesizing (R)-propoxybenzene derivatives using this enzyme requires addition and regeneration of NADPH, which is expensive and chemically unstable, and is industrially disadvantageous.

SUMMARY

An objective of the present invention is to provide novel enzymes that can reduce 4-haloacetoacetic acid esters using NADH as a coenzyme and produce (S)-4-halo-3-hydroxybutyric acid esters having high optical purities. Furthermore, an objective of the present invention is to provide methods for producing, using the enzyme, (S)-4-halo-3-hydroxybutyric acid esters having high optical purities.

In addition, an objective of the present invention is to provide novel enzymes that can produce optically highly pure (R)-propoxybenzene derivatives, which are useful as intermediates in synthesizing antibacterial drugs, using NADH as a coenzyme. Furthermore, an objective of the present invention is to provide methods for producing, using the enzyme, (R)-propoxybenzene derivatives that have high optical purities.

The present inventors thought that alcohol dehydrogenase that can use NADH as an electron donor was useful for industrial use. NADH is cheaper and chemically more stable

than NADPH. To discover enzymes that can effectively produce optically active (S)-4-halo-3-hydroxybutyric acid esters, the present inventors screened for alcohol dehydrogenase which has high activity on (R)-2-octanol, which has the same configuration as that of (S)-4-halo-3-hydroxybutyric acid esters and which has long chain as long as that of 4-haloacetoacetic acid esters.

Previous findings reported the enzymes derived from *Comamonas terrigena*, *Pichia sp.* NRRL-Y-11328, and *pseudomonas sp.* SPD6 as secondary alcohol dehydrogenases that can oxidize (R)-2-octanol stereoselectively and have activities to produce 2-octanone. However, no report has been made that these enzymes can reduce 4-haloacetoacetic acid esters and produce (S)-4-halo-3-hydroxybutyric acid esters. Activities to produce (S)-4-halo-3-hydroxybutyric acid esters by reducing 4-haloacetoacetic acid esters whose carbonyl group is bound to bulky side chains are expected to be low because activities of these enzymes for (R)-2-octanol are not significantly higher than activities for secondary alcohol like 2-propanol, which has short side chains.

Therefore, the present inventors screened widely for microorganisms that possess enzymes having ability to oxidize (R)-2-octanol preferentially. As a result, they have discovered that the microorganisms belonging to the genera below possess enzymes having ability to oxidize (R)-2-octanol preferentially:

Genus *Pichia*

Genus *Candida*

Genus *Ogataea*

Specifically, microorganisms below are found to possess enzymes having ability to oxidize (R)-2-octanol preferentially.

Pichia finlandica

Pichia jadinii

Candida utilis

Ogataea wickerhamii

Moreover, the present inventors cultivated these microorganisms and purified enzymes that can oxidize (R)-2-octanol from the microorganisms. As a result of examination of properties of these enzymes, the enzymes were found to oxidize (R)-2-octanol highly stereoselectively and, furthermore, to oxidize many secondary alcohols other than